

# EXHIBIT E

Directed cytokine expression in tumour cells  
*in vivo* using recombinant vaccinia virus

B. Acres  
K. Dott  
L. Stefani  
M.-P. Kieny

Department of Immunology,  
Transgene SA, 11 rue de Molsheim,  
Strasbourg,  
France

**Summary**

Athymic (Swiss nude) and euthymic (DBA) tumour-bearing mice were injected intravenously with various vaccinia virus (Copenhagen strain) recombinants. Several days after inoculation, tumour cells were found to be well infected with infective vaccinia particles, while organs such as liver, spleen, brain and bone marrow showed barely detectable levels or no signs at all of virus infection. Injection of tumour bearing mice with recombinant VV harbouring the cDNA for either *hIL-2* or *mIL-6* resulted in detectable lymphokine in the sera of injected animals. Injection of tumour-bearing nude mice with VV-IL-6, but not with VV-IL-2, resulted in significant reduction in growth rate of the tumour, and in some cases, complete rejection of the tumour. Tumour-bearing euthymic mice responded differently. Intravenous injection of VV-IL-2, but not VV-IL-6 resulted in reduced growth rate of 50% of tumours and complete rejection of 17% of tumours.

**Keywords**

cytokines, gene therapy, tumour, vaccinia virus

**Introduction**

Cytokines have proven to be useful biological response modifiers in the treatment of some forms of cancer (1). With the availability of purified recombinant cytokines clinical protocols involving the injection of lymphokines into patients has become feasible, although expensive. Cytokines often act locally, at the sites of inflammation or interactions between lymphocytes, with high regional concentrations but which diffuse rapidly such that the lymphokine is undetectable systemically. However, in therapy large doses must be administered systemically to produce the desired effect. This often results in serious unwanted side-effects (1-3).

Recent experimental evidence has demonstrated that tumour cells transfected with cDNA coding for cytokines will produce those cytokines when the cells are injected into experimental animals. In some cases, such as with tumours producing IL-1, IL-2, IL-4, IL-6, IL-7, IFN, TNF, G-CSF or GM-CSF, tumours are subsequently rejected (4-16), often with the development of tumour specific immunity such that if the same tumour cells, not secreting cytokines, are reimplanted the tumour is rejected. Clinical applicability of these observations is however, restricted to surgically accessible tumours and to tumours from which cell lines can be established. Furthermore the re-injection of tumour cells, even if transfected with lymphokines, may not always be desirable. Clearly, a method for the targeted delivery of lymphokine expression vectors to tumours is a goal worth pursuing.

Viral vectors have strong potential as vehicles for the targeted delivery of cytokine expression to tumour cells *in vivo*. Candidate viruses include retroviruses, adenovirus and poxviruses. Poxviruses, such as vaccinia virus, have several advantages: for example, the poxvirus genome can accommodate the insertion of relatively large fragments of DNA (17) into a choice of sites, and they are relatively easy to manipulate and purify. Moreover, infections are usually self-limiting. There has been some concern over the safety of recombinant vaccinia virus. However, there have been several advances in knowledge of its pathogenicity. We have used recombinants of the Copenhagen strain (18) of VV which has been shown to be 5000 times less virulent than the laboratory strain WR when injected intracranially into 3-week-old mice and it is better tolerated by nude mice ( $LD_{50}$  has been shown to be  $> 10^8$  PFU as opposed to 422 for WR) (19). Furthermore, mutations in the thymidine kinase gene of vaccinia drastically reduces virulence probably by making virus much less infective for quiescent cells (20,21). The vaccinia recombinants described in this report have all used the TK gene as the site of integration for the recombinant cDNA, thus rendering the virus TK-.

In this paper we report the results of experiments which show that the intravenous injection of control TK- or recombinant vaccinia virus into nude or euthymic tumour-bearing mice results in a high rate of infection of tumour cells while internal organs remain either uninfected or infected for a brief period and at a very low rate. We have taken advantage of this observation to determine whether vaccinia virus can be used to express cytokine cDNA in

tumour cells and to determine the effect on tumour development.

## Methods

### Mice

Female Swiss nude and DBA/2 mice were purchased from Iffa Credo (Lyon, France) and housed for at least one week prior to their use in an experiment. Mice were 6–8 weeks of age at the initiation of each experiment. Mice injected with vaccinia virus were housed in a P3 containment facility. Intravenous injection of VV was with a 27-gauge needle and by tail vein after warming the mice with a 60 watt lamp.

### Vaccinia recombinants

The cDNA corresponding to human IL2 was cloned at Transgene. The cDNA corresponding to murine IL-6 was from Immunex (Seattle, USA). The cDNAs were cloned into the thymidine kinase gene of the Copenhagen strain of vaccinia virus as described elsewhere (19). Expression of the IL2 and IL6 cDNAs are under the control of the 7.5 kDa promoter (19). Control viruses were either VV-186 which is TK- but has no insert, or VVi which is TK- and has an irrelevant insert.

### Assay for VV infection of tumours and organs by the infectious centre assay

Nude or DBA mice were injected with  $10^7$  SW948 or  $10^5$  P815 cells respectively in 100  $\mu$ l of PBS (Dulbecco's Phosphate Buffered Saline, Sigma D5652) using a 27-gauge needle, passing under the skin for at least 1 cm before implanting tumour cells. Seven days later tumours were palpable and mice were injected with 100  $\mu$ l PBS containing VV at the dosage indicated or 100  $\mu$ l of PBS alone. After the indicated time, mice were sacrificed by  $\text{CO}_2$  asphyxiation and tumours and organs removed. Also, 200  $\mu$ l of blood was collected and mixed with 200  $\mu$ l PBS plus 20-mm EDTA and assessed for viral content where indicated. Organs and tumours were disrupted mechanically with scissors and forceps plus repeated pipetting in 0.05% trypsin until a single cell suspension was obtained. Cells were washed twice with PBS, resuspended in tissue culture medium and stored overnight at 4°C. The following day cells were counted (both viable and nonviable) and dilutions made. Serial ten-fold dilutions, in duplicate, were made and added to established cultures of BHK cells in 3-cm-diameter Petri dishes (Falcon 3001) in 100  $\mu$ l, 10  $\mu$ l or 1  $\mu$ l volumes. Dishes were then incubated overnight at 37°C, 5%  $\text{CO}_2$  and plaques counted the following day.

### Cytokine bioassays

IL-2 bioactivity was assessed using the CTLL proliferation

assay (22). Serum samples were diluted serially in 96-well flat-bottomed microculture plates (Falcon 3072) in RPMI 1640 medium supplemented with 10% selected fetal bovine serum, glutamine, pyruvate, nonessential amino acids and 50- $\mu$ M 2-mercapto-ethanol. CTLL cells were taken from continuous culture in the same medium containing 10 ng  $\text{ml}^{-1}$  recombinant human IL-2. Cells were washed at least three times by centrifugation and incubated for 3 h at 37°C without IL-2. Cells were then washed twice, counted and 5000 cells per well added to the plates at a final volume of 100  $\mu$ l. Positive control consisted of 10 ng  $\text{ml}^{-1}$  rIL-2 also diluted serially. Negative controls were normal mouse serum (either nude or DBA where applicable) and no stimulant at all. Cells were incubated for 48 h and 1  $\mu$ Ci 3H-Thymidine (Amersham) per well added for the final 4 h of culture. Cells were then harvested onto glass-fibre paper using a PHD Mash harvester and samples counted by liquid scintillation. Data was plotted and the dilution required for 50% maximal proliferation was taken as having 1 unit of activity;  $\text{U ml}^{-1}$  could then be easily calculated. Positive control rIL-2 routinely had a specific activity of between 3 and 10 pg  $\text{ml}^{-1}$  for 1 U of activity.

IL-6 bioassay was essentially the same with the exceptions that 7TD1 cells (kindly supplied by Dr Jacques van Snick, Ludwig Institute, Brussels) were added rather than CTLL and the culture medium for the assay was Dulbecco's modified Eagles medium supplemented with 5% FBS and other supplements described above. 7TD1 cells were maintained in culture in the same medium as for CTLL with the exception that medium was supplemented with 10 ng  $\text{ml}^{-1}$  human recombinant IL-6. Specific activity of rIL-6 is routinely between 1 and 3 pg  $\text{ml}^{-1}$  corresponding to 1 unit of activity.

### Tumour cells

The human colorectal tumour cell line SW948 (ATCC CCL 237) and the murine DBA mastocytoma cell line P815 (ATCC TIB 64) were purchased from ATCC. Cells were taken from continuous culture immediately prior to injection into mice. SW948 cells were trypsinized and treated with 10  $\mu$ g  $\text{ml}^{-1}$  DNase (Sigma) in culture medium for five minutes to eliminate viscosity and loss of cells due to liberation of DNA from dead cells during the trypsinization process. SW948 and P815 cells were washed in PBS and counted. Cells were resuspended in PBS at concentrations suitable for injecting the indicated number of cells in a 100- $\mu$ l volume.

### Tumour measurement

Palpable tumours were measured with callipers (Mitutoyo, Japan). Measurements of length, width and thickness of tumours were taken and tumour volume calculated by the formula  $4/3\pi r_1 r_2 r_3$ .

## Results

### In-vivo infection of tumour cells by VV

Deletion or mutation of the TK gene renders VV much less infective for the CNS and probably other quiescent cells (20,21) while remaining infectious for rapidly dividing cells. Thus it was reasoned that a TK- Copenhagen virus should infect preferentially tumour tissues when injected into mice with tumours. To test this hypothesis, various recombinant as well as a control TK- VV (VV-186) were injected intravenously into tumour-bearing nude and DBA mice. Tumour-bearing nude mice were carrying palpable tumours resulting from the injection of the human colorectal tumour cells SW948 as described in Methods. Tumour-bearing DBA mice carried palpable tumours resulting from the injection of DBA P815 mastocytoma tumour cells. Their tumours and various organs were then analysed for viral infection at various intervals after virus injection. The results are shown in Table 1. In nude mice, tumours cells are 100% infected one week after administration of 10<sup>8</sup> PFU of all three VV constructs tested. At this dose, no evidence of infection of peripheral blood mononuclear cells was found and very little virus was found in the spleen. However, at this dose of virus, some nude mice become dehydrated and unhealthy in appearance. The  $LD_{50}$  for the Copenhagen strain of VV has been reported to be greater than 10<sup>8</sup> PFU in nude mice (19) so the sickly appearance of some of the tumour-bearing nude mice could be due to the dose approaching the  $LD_{50}$  or due to production of toxic lymphokines such as Tumour Necrosis Factor (1). The experiment was repeated using ten fold less virus. At the dose of 10<sup>7</sup> PFU, nude mice remained healthy and content in appearance. At this dose almost 100% of tumour cells are infected and no detectable infection of the spleen and liver was observed, but a minor infection of bone marrow cells was seen, depending on the construct tested. The cytokine cDNA-carrying constructs showed less infection of bone marrow cells than did control constructs VV-186 and VVi. Rapid clearance of VV-IL-2 from nude mice has been demonstrated elsewhere (23-25) and some T-cell maturation in nude mice as a result of injection with VV-IL6, which may also aid in viral clearance, has also been shown

(26). Previous studies have shown that IV injection of euthymic mice with VV results in evidence of virus peaking between days 3 and 4 after injection, after which virus is rapidly cleared (26). Tumour-bearing DBA mice were injected IV with 10<sup>8</sup> PFU of various VV constructs. Tumours, various organs and blood were subsequently tested for evidence of viral infection (Table 2). At this dose of virus no ill health was observed in DBA mice and no infection of brain cells was observed at three days after injection of virus. Some barely detectable infection of spleen cells was observed, in agreement with previous observations (26). At day 3 tumour cells were about 2% infected except for cells from mice injected with VV-IL2, which showed a lower but still significant rate of infection. Interestingly, the opposite was observed in the liver in that the most infection was observed in the VV-IL2-injected mice. This suggests that the VV-IL2 construct is cleared more rapidly and, moreover, that the liver is likely the site of viral clearance. After 4 days, infection of tumour cells while still much greater than

**Table 2** VV titre in organs and tumours of tumour-bearing DBA (euthymic) mice after intravenous VV injection. Female mice, 6-8 weeks of age, were injected subcutaneously with 10<sup>8</sup> P815 tumour cells in 100 µl of PBS. One week later tumours were palpable. At this point mice were injected IV with 10<sup>8</sup> PFU VV in 100 µl of PBS. On the days indicated mice were sacrificed and tumours and organs assessed for viral infection as described. Two to four mice per group were assessed and the range of viral titres for each group shown where a titre was detected

VV injected*	VV titre (PFU/10 <sup>8</sup> cells)				
	Tumour	Spleen	Liver	BM	Brain
Day 3					
VV-186	(1.5-4.8) × 10 <sup>4</sup>	< 1	< 1	< 0.1	< 1
VV-IL2	350-900	< 1	< 1-12	< 0.1	< 1
VV-IL6	(0.2-2.3) × 10 <sup>4</sup>	< 1	< 1-2	< 0.1	< 1
Day 4					
VV-186	40-1350	0.4	1-11	ND	ND
VV-IL2	65-150	0.2	< 1	ND	ND
VV-IL6	280-400	0.2	< 1	ND	ND
Day 7					
VV-186	700-4000	< 5	< 5	< 5	ND
VV-IL2	< 5	< 5	< 5	< 5	ND

\*Days after IV injection with VV.

**Table 1** Titre of VV-infected cells in organs and tumours of tumour-bearing nude mice after iv VV injection. Female Swiss nude mice, 6-8 weeks of age, were injected subcutaneously with 10<sup>7</sup> SW948 tumour cells in 100 µl of PBS. One week later tumours were both visible and palpable. At this point mice were injected iv with 10<sup>8</sup> or 10<sup>7</sup> PFU VV in 100 µl of PBS or 100 µl of PBS alone. Seven days later mice were killed and tumours and organs assessed for viral infectious centres as described. Two to four mice per group were assessed. The range of PFU titres are shown

VV injected	VV titre (PFU/10 <sup>8</sup> cells)				
	Tumour	PBL	Spleen	Liver	BM
10 <sup>8</sup> PFU VVi	(10-23) × 10 <sup>5</sup>	< 1	0.5-9	ND	< 1
10 <sup>8</sup> PFU VV-2179	(10-15) × 10 <sup>5</sup>	< 1	< 1-2	ND	< 1
10 <sup>8</sup> PFU VV-1142	(22-23) × 10 <sup>5</sup>	< 1	1-2	ND	< 1
10 <sup>7</sup> PFU VV-186	(1.2-14) × 10 <sup>5</sup>	ND	< 1	< 1	< 1-17
10 <sup>7</sup> PFU VVi	(0.2-10) × 10 <sup>5</sup>	ND	< 1	< 1	< 1-25
10 <sup>7</sup> PFU VV-IL2	(0.9-1.0) × 10 <sup>5</sup>	ND	< 1	< 1	< 1-1
10 <sup>7</sup> PFU VV-IL6	(0.5-1.2) × 10 <sup>5</sup>	ND	< 1	< 1	< 1

ND = not done.

that of organs, fell rapidly. Infection of tumour cells by the control VV-186 continued to at least day 7 after injection of virus.

#### In-vivo cytokine production in mice after injection with VV-cytokines

Tumour-bearing nude and DBA mice were injected IV with  $10^8$  PFU VV-186, VV-IL2, or VV-IL6 and their sera monitored for IL-2 and IL6 content by bioassay. BHK cells infected with VV-IL2 or VV-IL6 produce 1–10  $\mu\text{g ml}^{-1}$  rIL-2 and IL-6, respectively in culture after a 24-h infection (data not shown). However, no evidence of IL2 activity was found in the serum of nude mice after injection of  $10^8$  PFU VV-IL2. That IL2 was being produced in these mice was probable since the virus was more rapidly cleared, and the mice appeared particularly healthy and robust, whereas mice injected with  $10^8$  PFU of the other constructs often appeared dehydrated, as described above. Both of these effects have been attributed to *in-vivo* IL2 production (23,24) and the mediators of this effect defined as NK cells (25). On the third day of viral infection of tumour bearing DBA mice, weak IL2 activity (30 U/ml) was detected in the sera of VV-IL2-injected mice but not in sera of VV-IL6 or control-injected mice. Serum IL6 was much more readily detected as shown in Table 3. Nude mice whose tumours are 100% infected at day 7 after VV injection (Table 1) show very high levels of IL6 activity after injection with VV-IL6, but not with VV-IL2 or control. Euthymic DBA mice show lower but still significant levels of serum IL6 which corresponds to the kinetics of infection.

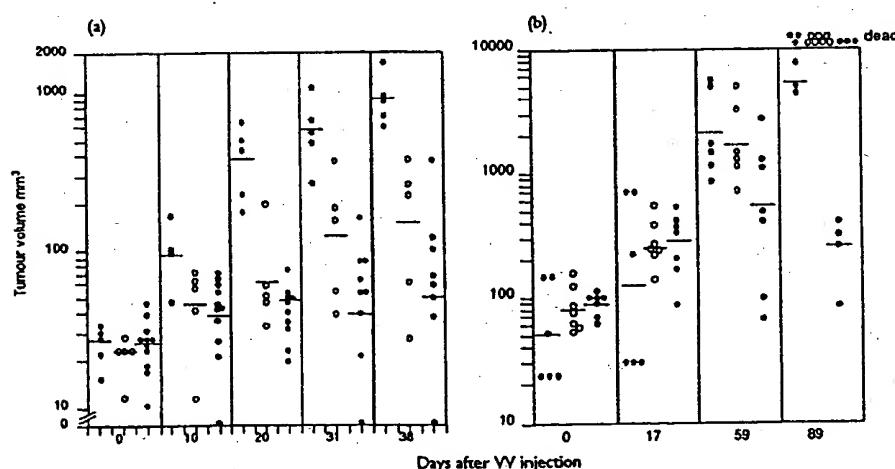
#### Effects of VV-IL6 on tumour growth in nude mice

The effects of injection of VV-IL2 and VV-IL6 on growth of a human tumour growing in nude mice were examined.

**Table 3** Serum IL6 levels after injection of VV-IL6 into tumour-bearing nude or DBA mice. Serum samples were assayed for IL6 content by bioassay using 7TD1 cells. Initial serum content was 1/50 and serial 1/2 log dilutions made in duplicate. Standard for the assay was 100  $\text{pg ml}^{-1}$  human recombinant IL-6. Fifty per cent maximum proliferation of 7TD1 cells occurred at 3  $\text{pg ml}^{-1}$  in both assays. The minimum detectable IL6 concentration was 1  $\text{pg ml}^{-1}$ . Thus where no 7TD1 cell proliferation occurred it was taken as < 50  $\text{pg ml}^{-1}$  IL6

injection	Serum IL6 ( $\text{pg ml}^{-1}$ )			
	day 1	day 3	day 4	day 6
<b>Nude mice</b>				
0	< 50	< 50	< 50	< 50
VV-186	< 50	< 50	< 50	< 50
VV-IL2	< 50	< 50	< 50	< 50
VV-IL6	143			9400
<b>DBA mice</b>				
0	< 50	< 50	< 50	< 50
VV-186	< 50	< 50	< 50	< 50
VV-IL2	< 50	< 50	< 50	< 50
VV-IL6	57	288	80	< 50

Female nude mice were injected subcutaneously with  $10^7$  SW948 tumour cells. After 7 days tumours were both visible and palpable. At this point, mice were injected with  $10^8$  (expt 1) or  $10^7$  (expt 2) PFU VV-IL6, negative control VVi or PBS alone. Tumour growth was then monitored and the results shown in Fig. 1. Tumours in the PBS injected group grew progressively with no gross evidence of invasion or metastasis. Mice in the  $10^8$  PFU VV injected groups eventually appeared dehydrated and weak with one mouse in the VV-IL6 group dying on day 20. Tumours continued to grow but more slowly than those on the PBS injected mice possibly as a result of dehydration. Nonetheless, tumours on mice injected with VV-IL6 were on average smaller than those on the control VV injected mice with one mouse completely rejecting its tumour. At day 38 mice were killed



**Fig. 1** Female Swiss nude mice, aged 6–8 weeks were injected sub-cutaneously with  $10^7$  SW948 tumour cells. After 1 week tumours were palpable and mice were injected IV with  $10^8$  (a) or  $10^7$  (b) PFU VVi (○), VV-IL6 (●) or 100  $\mu\text{l}$  of PBS (◊). Subsequent tumour growth was monitored by measuring length, width and thickness of the tumours on the days shown. Tumour volume was calculated as described in Methods.  $\chi^2$  analysis of the tumour sizes at day 89 indicate data VV-IL6 has a significant effect on tumour growth ( $P < 0.05$ ).

and their serum tested for SW948 specific antibody by FACS analysis, and their spleen cells tested for cytotoxicity towards SW948 cells. No evidence of either was observed (data not shown). Since nude mice have been observed to support infection better with  $10^7$  rather than  $10^6$  PFU VV, the experiment was repeated with the lower dose of virus. The results are shown in Fig. 1(b). No effect of control VV on tumour growth was seen. In contrast, injection of tumour bearing mice with  $10^7$  PFU VV-IL6 retarded tumour growth in more than half of the VV-IL6 injected group. At day 100 surviving mice were sacrificed and their tumours assessed for VV infection. No evidence of infection was found.

It has been reported that nude mice injected with VV-IL2 have increased NK activity (25), however, no evidence of tumour rejection or retardation of tumour growth was observed in tumour bearing nude mice injected with VV-IL2 (data not shown).

#### Effect of VV-cytokines on tumour growth in euthymic mice

Despite the relatively low infection rates of VV infection of P815 tumour cells in DBA mice after VV injection, it was decided to assess the effect of VV-cytokines on *in-vivo* tumour growth. DBA mice with palpable P815 tumours were injected IV with vaccinia-cytokine constructs, control vaccinia or PBS alone. Tumour growth was monitored and the results of one experiment shown on Fig. 2. Two of the four mice injected with VV-IL2 rejected their tumours, whereas all mice in the control groups had continuously growing tumours until sacrifice at day 21. Serum was tested for anti-P815 antibody titre by incubating serial dilutions of serum

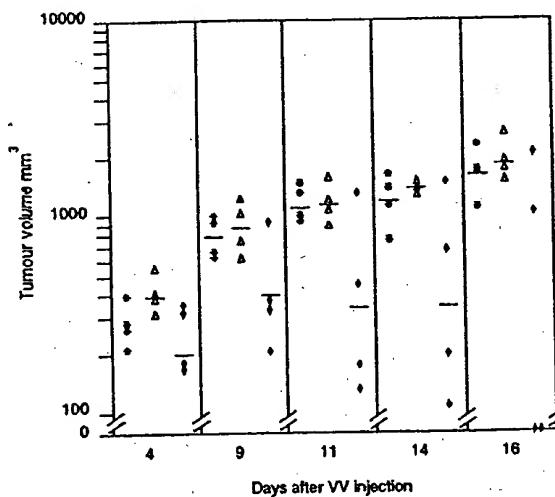


Fig. 2 Female DBA/2 mice were injected subcutaneously with  $3 \times 10^6$  P815 tumour cells. After 1 week tumours were palpable and mice were injected with  $10^7$  PFU VV-186 ( $\Delta$ ),  $10^7$  pfu VV-IL2 ( $\diamond$ ) or  $100 \mu\text{l}$  PBS ( $\square$ ). Tumour growth was monitored by measuring tumour length, width and thickness on the days shown. Tumour volume was calculated as described in the Methods.

Table 4 Frequency of tumour rejection or growth retardation by tumour-bearing DBA/2 mice injected IV with VV-IL2. Combined results of eight experiments. DBA/2 female mice were injected SC with P815 tumour cells. In four experiments,  $10^6$  P815 tumour cells were injected. In other experiments, doses of  $10^7$ ,  $3 \times 10^6$  and  $10^6$  cells were assessed and no difference in outcome was observed to correlate with the number of tumour cells injected. When tumours were easily palpable ( $c. 8 \text{ mm} \times 5 \text{ mm} \times 5 \text{ mm}$ ) mice were injected intravenously with  $10^7$  PFU VV-IL2,  $10^7$  pfu VV-control or  $100 \mu\text{l}$  PBS and tumours then measured twice weekly.  $\chi^2$  analysis indicates that the frequency of tumour rejection induced by VV-IL2 is statistically significant ( $P < 0.025$ ) as are the frequency of slower tumour growth ( $P < 0.025$ ) and the combined frequencies indicating significant biological effect ( $P < 0.001$ )

Injected with:	VV-IL2	VV-Control*	PBS
Tumour rejected†	7/42 (17%)	0/41 (0%)	0/12
Slower tumour growth‡	14/42 (33%)	1/41 (2%)	0/12
Combined frequency§	21/42 (50%)	1/41 (2%)	0/12

\* VV-Control = VV with no insert or with an irrelevant insert;

† tumours disappeared after injection of virus, and did not reappear for the life of the animal

‡ size of the tumour was at least two standard deviations smaller than those in either control group at the time of measurement

§ (i.e. significant effect on tumour growth) frequencies of tumours rejected and slower tumour growth are added together to show the frequency of mice showing a significant effect of VV-IL2 administration on tumour growth

with P815 cells and assessing bound antibody by FACS analysis. Mice whose tumours had regressed had a ten-fold higher titre of anti-P815 antibody by this assay (data not shown). However, it is difficult to say at this point whether this is the cause or result of the tumour rejection.

The results of several experiments (including expt 2, the results of which appear in Fig. 2) are summarized in Table 4. Injection of VV-IL2 significantly retarded tumour growth on one-half of the mice injected with VV-IL2 and 17% of mice rejected their tumours completely. Injection of lower doses of virus,  $10^7$  or  $10^6$  PFU had no effect on tumour growth (data not shown).

#### Discussion

The experiments described in this manuscript demonstrate that tumour cells can be efficiently infected with vaccinia virus *in vivo*, while other organs remain much less infected. This is possibly explained by the previous observation that TK- mutants of VV are much less able to infect quiescent cells *in vivo*. The liver, which is the site of viral destruction, shows a low level and transient infection. That tumour cells are infected *in vivo* by VV allows the transient expression of foreign genes in tumours.

Vaccinia virus has been used previously to express cytokines *in vivo* to observe their biological effects (23-26). In agreement with Nakagawa *et al.* (26), we find that murine IL6 expressed in nude and euthymic mice results in detectable IL6 activity in the serum which corresponds to

the duration of the VV infection. IL6 has been observed in human serum (27) and has been shown to have many pleiotropic effects (28), suggesting that IL6 can act systemically and that it is well tolerated when in circulation. IL2, on the other hand, functions locally at the site of lymphocyte interactions. Endogenously produced IL-2 is not found in serum and is not well tolerated when injected systemically in large doses (2,3). Injected IL2 has a half life of two to three minutes when injected into experimental animals (29), suggesting that this cytokine is rapidly destroyed when it reaches the circulation. When nude mice were injected with VV-IL2 no circulating IL2 could be found in the serum during the course of the viral infection, and when euthymic mice were injected only weak and transient activity was found. Nonetheless, *in-vivo* effects of both cytokines were observed.

In nude mice, VV-IL2 has been reported to enhance natural killer (NK) cell activity (25) while injection of VV-IL6 has resulted in some T-cell maturation (26). VV-IL2 was not able to affect the growth of tumours in nude mice (data not shown) suggesting that NK cell activity was not enough to induce the rejection of these tumours. Interestingly, VV-IL6 did cause retarded tumour growth and occasionally tumour rejection in nude mice. In euthymic mice the reverse was true in that VV-IL6 had little or no effect on tumour growth (data not shown) and VV-IL2 caused the retardation of growth and some rejection of tumours.

It is unfortunate, for these purposes, that VV-IL2 is cleared so rapidly, likely due to the local effects of IL2 in the anti-VV immune response. Nonetheless, infection of tumour cells, even though low level and transient, was enough to provoke some antitumour response resulting in either retardation of growth or outright rejection of P815 tumours. Alternate recombinant viruses, such as avian poxvirus or NYVAC which infect mammalian cells and produce recombinant protein, but do not replicate (19) may infect tumours and thus could also be considered as vectors for delivery of cytokine expression to tumours *in vivo*. Avian poxvirus would have the additional advantage that an existing immunity to avian pox virus is highly unlikely in cancer patients. For this purpose a transient expression system such as a poxvirus would be preferable to retroviruses since some infection of normal tissues was observed. Thus a recombinant retrovirus, which results in permanent expression due to recombination with the host genome, would be undesirable as it would result in permanent cytokine secretion by infected normal as well as tumour cells. A third viral option is adenovirus, which exists episomally in infected cells, resulting in the expression of recombinant protein, but appears not to be passed on to daughter cells upon cell division (31), so would eventually be diluted out.

Other cytokines such as TNF which would affect tumour cells directly, or GM-CSF which has recently been shown to be effective in a murine tumour model system (16) should also be assessed in a viral delivery system. In pilot experiments (data not shown) we have assessed in our

two tumour model systems, VV-murine-IL4 and VV-murine IL5. They appear not to affect tumour growth.

The observations reported in this manuscript lend credibility to the concept of gene therapy for cancer treatment. Targeted delivery of cytokine expression to tumours *in vivo* is obtainable with a viral vector. In a human tumour model as well as a murine tumour system this approach was shown to modify tumour growth. This process provides a viable alternative to the process of injecting large quantities of recombinant cytokines for treatment of cancer.

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